

# Prokineticin 2 Is a Target Gene of Proneural Basic Helix-Loop-Helix Factors for Olfactory Bulb Neurogenesis<sup>\*[S]</sup>

Received for publication, November 15, 2006, and in revised form, January 17, 2007  
Published, JBC Papers in Press, January 26, 2007, DOI 10.1074/jbc.C600290200

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Prokineticin 2, a cysteine-rich secreted protein, regulates diverse biological functions including the neurogenesis of olfactory bulb. Here we show that the *PK2* gene is a functional target gene of proneural basic helix-loop-helix (bHLH) factors. Neurogenin 1 and MASH1 activate *PK2* transcription by binding to E-box motifs on the *PK2* promoter with the same set of E-boxes critical for another pair of bHLH factors, CLOCK and BMAL1, in the regulation of circadian clock. Our results establish *PK2* as a common functional target gene for different bHLH transcriptional factors in mediating their respective functions.

The olfactory bulb (OB)<sup>3</sup> is one of the few structures that have a continuous supply of newly generated neuron in the adult brain (1, 2). Neuroprogenitor cells arise from the stem cells located in the anterior subventricular zone of the lateral ventricle (3, 4) and migrate tangentially as homotypic cell chains, using each other as their migration substrate, into the OB (5, 6), where they differentiate into inhibitory interneurons in the granule cell layer (GCL) and periglomerular layer (PGL) (7).

A set of proneural genes encoding the bHLH transcription factor family is crucial for the development of vertebrate and invertebrate nervous systems (8, 9). These bHLH factors exert their functions by binding to E-box motifs (CANNTG) on the

promoters of their target genes (9). During development, proneural bHLH factors such as Neurogenin 1 (Ngn1) and MASH1 regulate several important neural developmental stages, including the commitment of stem cells to neuronal and glial lineages (10, 11), the specification of neuronal subtype identities (12, 13), and neuronal migration (14, 15).

Prokineticin 2 (PK2) regulates various biological functions, including angiogenesis (16, 17), circadian rhythm (18), and neurogenesis in the adult OB (19) via the activation of two cognate G-protein-coupled receptors (20). In the adult mouse brain, PK2 is expressed abundantly in the suprachiasmatic nucleus (SCN) and the GCL and PGL in the OB (18, 19, 21). In the SCN, a heterodimer of CLOCK and BMAL1, two bHLH factors, regulates the rhythmic expression of the *PK2* gene by binding to E-box elements on the *PK2* promoter (18). *PK2* expression in the SCN is abolished in *Clock*<sup>−/−</sup> and *Bmal1*<sup>−/−</sup> mice (18), supporting the role of *PK2* in regulating circadian rhythm as a target gene of CLOCK and BMAL1. However, the transcriptional regulation of the *PK2* gene in the OB remains unknown. The abnormal OB development in *PK2*-deficient mice (19) and normal OB in *Clock*<sup>−/−</sup> and *Bmal1*<sup>−/−</sup> mice (22, 23) indicates that PK2 is likely under the control of bHLH factors other than CLOCK and BMAL1 in the OB.

In this study, we examine the regulation of the *PK2* gene by the proneural bHLH factors Ngn1 and MASH1 in the embryonic and postnatal mouse OB. We found that the expressions of these genes overlap in several populations of OB neurons, and the expression of the *PK2* gene is reduced in Ngn1 and MASH1 mutant mice. Both Ngn1 and MASH1 positively regulate the *PK2* gene transcription by binding to E-box motifs on the *PK2* promoter. Moreover, both PK2 and Ngn1 mutant mice have similar defects in OB development. Our results indicate that *PK2* is a target gene of proneural factors for neurogenesis in the OB.

## EXPERIMENTAL PROCEDURES

**Animals and Histological Examination**—Postnatal and adult mice were perfused with saline and 4% paraformaldehyde (PFA) under anesthesia. For the embryos, the day on which the vaginal plug was found was counted as embryonic (E) day 0.5. E13.5–E18.5 embryos were collected from pregnant mice by cesarean section. Embryos older than E15.5 were perfused transcardially. Cresyl violet staining on cryosections was carried out for histological examination. All procedures regarding the care and use of animals were in accordance with institutional guidelines.

**In Situ Hybridization**—Riboprobes for *PK2*, *Ngn1*, and *Mash1* were labeled with <sup>35</sup>S-UTP or digoxigenin (DIG)-UTP. *In situ* hybridization was performed on cryosections as described (18, 24). Relative density of hybridization signals using <sup>35</sup>S-labeled probes were analyzed with ImageJ from NIH.

**Immunohistochemistry**—Cryosections were blocked with 10% horse serum and incubated with primary antibody at 4 °C overnight. Fluorescent secondary antibodies (Jackson ImmunoResearch Laboratories, 1:200) were added subsequently. Sec-

<sup>\*</sup> The work was supported in part by a University of California Discovery grant. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>[S]</sup> The on-line version of this article (available at <http://www.jbc.org>) contains four supplemental figures.

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<sup>3</sup> The abbreviations used are: OB, olfactory bulb; PK2, prokineticin 2; bHLH, basic helix-loop-helix; Ngn1, Neurogenin 1; SCN, suprachiasmatic nucleus; GCL, granule cell layer; PGL, periglomerular layer; MCL, mitral cell layer; DIG, digoxigenin; ChIP, chromatin immunoprecipitation; P0, postnatal day 0; EGF, epidermal growth factor; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; WT, wild type; TH, tyrosine hydroxylase; PFA, paraformaldehyde.

tions were counterstained with 4',6-diamidino-2-phenylindole and viewed with a Zeiss fluorescence microscope.

Antibody used are: mouse anti-tyrosine hydroxylase (Novus Biologicals, 1:1000), rabbit anti-MASH1 (Chemicon, 1:100), mouse anti-Tuj1 (Covance, 1:200), rabbit anti-Ngn1 (gift from Michael Greenberg, 1:500). The specificity of Ngn1 antibody was tested by immunostaining on *Ngn1*<sup>-/-</sup> tissues.

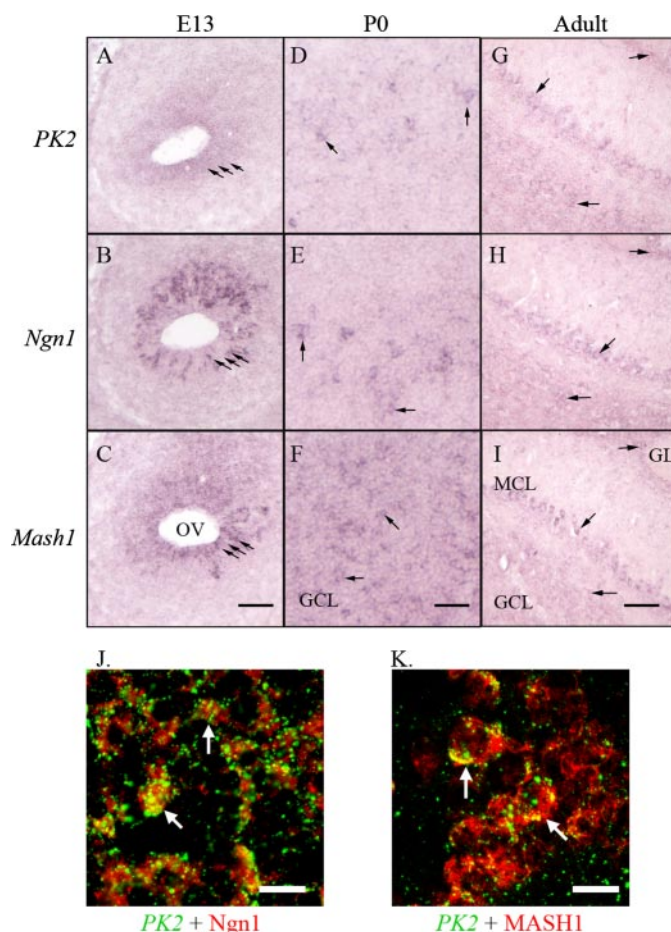
**Combo Immunostaining with *in situ* Hybridization**—*In situ* hybridization was performed with a DIG-labeled probe. Sections were then incubated in hydrogen peroxide to quench endogenous peroxidase activity, blocked in 10% horse serum, and incubated with an horseradish peroxidase-conjugated anti-DIG antibody (PerkinElmer Life Sciences, 1:500) at 4 °C overnight. *PK2*-expressing cells were revealed with the TSA Plus fluorescence kit (PerkinElmer Life Sciences). The sections were then immunostained and viewed under a confocal microscope.

**Luciferase Reporter Assay**—HEK293 cells were plated in 12-well plates at  $2 \times 10^5$  cells/well and transfected with Lipofectamine (Invitrogen). In all transfection assays, 100 ng of promoter constructs were used with 300 ng of expression constructs of Ngn1, MASH1, p300, or CBP. Cells were harvested after 36 h and analyzed for luciferase activity. Relative promoter activity was calculated as described (25).

**Chromatin Immunoprecipitation (ChIP)**—ChIP assay was performed using an EZ-ChIP kit (Upstate Biotechnology) following the provided instructions. Tissues from wild-type (WT) or mutant mice were minced with scalpels, fixed in 1% PFA at room temperature for 15 min, and homogenized. Chromatin was released by SDS lysis buffer and sonicated to fragments ranging from 200 to 1000 bp. Antibodies against MASH1, Ngn1, and RNA polymerase II were used for immunoprecipitation. Negative controls were performed with nonspecific IgG antibody, no DNA input, and tissues from *Ngn1*<sup>-/-</sup> and *Mash1*<sup>-/-</sup> mice. Primers used for PCR are as follows: E-box 1, 5'-TCTCAGCCTCG-GATACTTCAC-3' and 5'-GTTTCACCTTCCCACTCCTG-3'; E-box 2, 5'-CTTGCCCTTGTCTTGTCTG-3' and 5'-CTCCTCCTTCGCCGTCCTGT-3'; E-box 3, 5'-GATCA-GAACCCCAACCA-3' and 5'-CCTGACCCTTCTGC-CCTGA-3'; E-box 4, 5'-TGCTGGCTAATGCCACATGC-3' and 5'-ACTTCCTGGTCCAGCCACCA-3'.

***In Vitro* Differentiation of Neural Stem Cell**—Neural stem cells were isolated from the anterior subventricular zone of adult mice as described (26) and maintained as neurosphere in serum-free Neurobasal-A medium supplemented with B27 (Invitrogen) and 20 ng/ml EGF. Neurospheres were dissociated into single cells with trypsin and plated on poly-D-lysine-coated chamber slides (Nalge Nunc) at 20,000 cell/cm<sup>2</sup>. Differentiation were induced by replacing the EGF medium with medium containing 0.5% fetal bovine serum with PK2 or retinoid acid. Culture media were replaced every other day. The cells were fixed in 4% PFA for 15 min and immunostained after culture.

**Neurosphere Assay**—Neural stem cells were plated in uncoated 96-well plates at 200 cell/well in Neurobasal-A medium supplemented with different combinations of B27, PK2, and EGF. Neurospheres were allowed to grow and counted after 7 days.



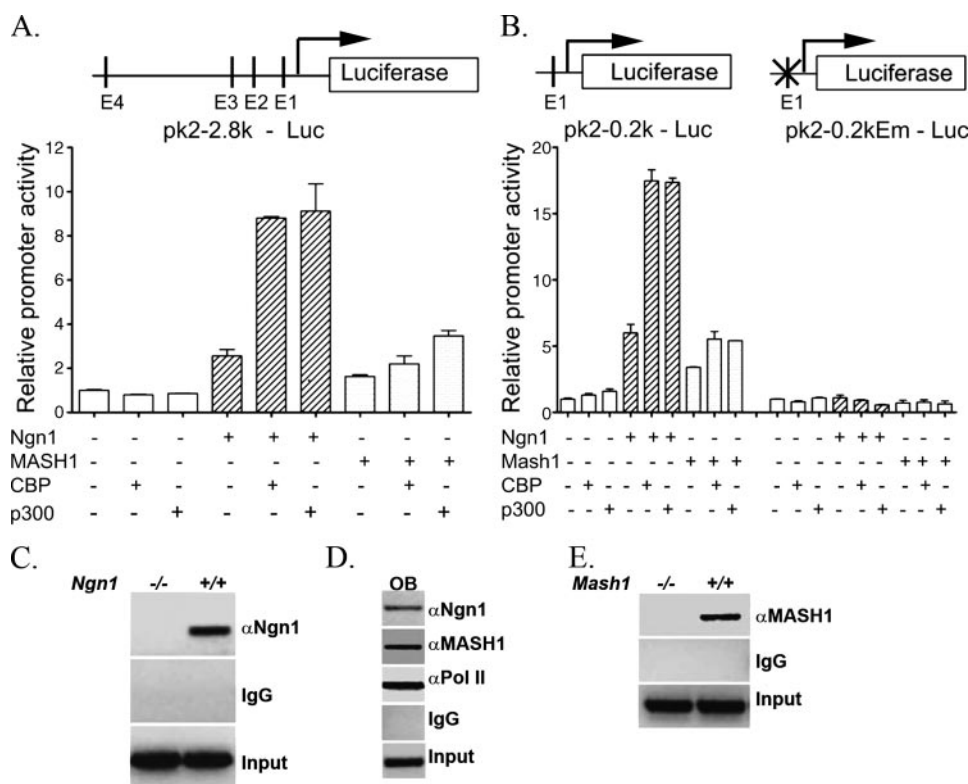
**FIGURE 1. bHLH factors and *PK2* expression in the OB.** A–I, *in situ* hybridization analysis of *PK2*, *Ngn1*, and *Mash1* expression in the OB of E13 (A–C), P0 (D–F), and adult (G–I) mice. Expressions of *PK2*, *Ngn1*, and *Mash1* overlapped in several populations of the neurons in the OB. Scale bar = 100 μm. OV, olfactory ventricle. GL, glomerular layer. J, co-localization of Ngn1 (red) and *PK2* (green) in neurons of the adult OB. K, co-localization of MASH1 (red) and *PK2* (green) in neurons of the adult OB. Scale bar = 15 μm.

## RESULTS AND DISCUSSION

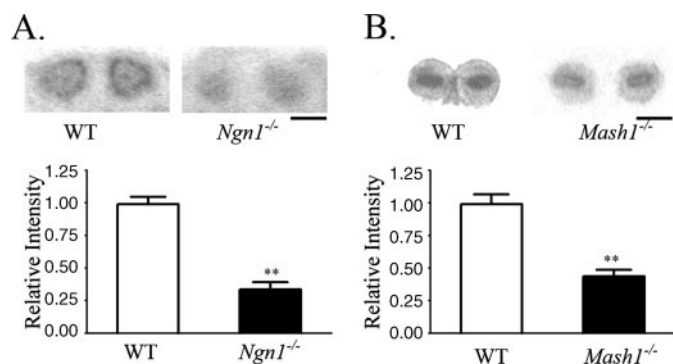
To test the hypothesis that proneural bHLH factors regulate *PK2* gene expression in the OB, we first examined the expressions of these genes in the OB of embryonic and postnatal mice. *In situ* hybridization with DIG-labeled antisense probe revealed that *PK2* was expressed in the ependymal and subependymal layers of the olfactory ventricle at E13. In postnatal day 0 (P0) and adult mice, *PK2* was expressed in the GCL, the PGL, as well as the mitral cell layer (MCL) (Fig. 1). Interestingly, similar expression patterns of *Ngn1* and *Mash1* were observed in the OB of E13, P0, and adult mice (Fig. 1 and supplemental Figs. 1 and 2; sense probes showed no hybridization signals, data not shown). The expression of *PK2*, *Ngn1*, and *Mash1* thus overlapped in several populations of OB neurons. Immunostaining against Ngn1 and *in situ* hybridization with DIG-labeled *PK2* probe showed that they were co-expressed in some neurons in the OB of adult and P0 mice. Similar co-localization was also observed for MASH1 and *PK2* (Fig. 1, J and K, and data not shown).

We then examined whether Ngn1 and MASH1 could activate the *PK2* promoter. As shown with luciferase reporter assays in HEK293 cells, heterologous expression of Ngn1 activated the *PK2* promoter moderately. A stronger activation was





**FIGURE 2. Ngn1 and MASH1 activate PK2 expression by binding to E-boxes on PK2 promoter.** A, luciferase (Luc) reporter assay of 2.8-kb PK2 promoter construct in HEK293 cells. Each assay was analyzed in triplicate. E1–E4 stand for four E-boxes on the PK2 promoter. B, luciferase reporter assay of wild-type 0.2-kb PK2 promoter construct and mutant E-box construct in HEK293 cells. C, a ChIP assay showed that Ngn1 associated with E1 motif in the WT P0 mice brain but not the *Ngn1*<sup>-/-</sup> mice. D, ChIP analyses demonstrated the association of Ngn1, MASH1, and RNA polymerase II (Pol II) with the E1 motif in the OB of adult mice. E, ChIP analyses showed MASH1 associated with the E1 motif in the WT E16 mice brain but not the *Mash1*<sup>-/-</sup> mice.



**FIGURE 3. Reduced PK2 expression in the OB of *Ngn1*<sup>-/-</sup> and *Mash1*<sup>-/-</sup> mice.** A, <sup>35</sup>S-labeled *in situ* hybridization of PK2 expression in the OB of P0 WT and *Ngn1*<sup>-/-</sup> mice and quantification of PK2 signals. \*\*,  $p < 0.01$ ,  $t$  test,  $n = 4$ . Scale bar = 1 mm. B, <sup>35</sup>S-labeled *in situ* hybridization of PK2 expression in the OB of E16.5 WT and *Mash1*<sup>-/-</sup> mice and quantification of PK2 signals. \*\*,  $p < 0.01$ ,  $t$  test,  $n = 3$ .

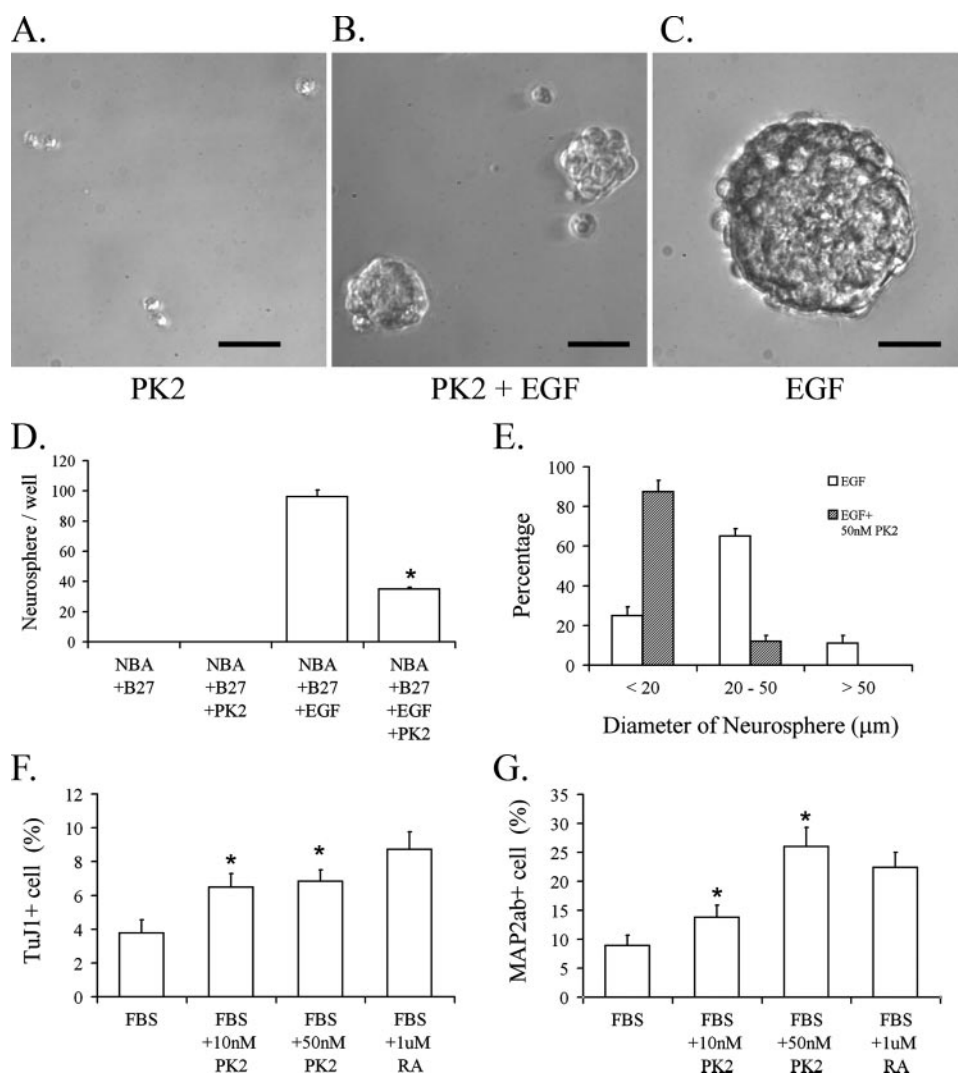
produced upon the addition of CBP or p300, two transcriptional coactivators of Ngn1 (11) (Fig. 2A). A construct containing the proximal E-box of the PK2 promoter could also be transactivated by Ngn1, with or without CBP and p300, whereas mutation of the E-box abolished the activation (Fig. 2B). Comparable with Ngn1, heterologous expression of MASH1 also transactivated the PK2 promoter, with moderate enhancement by CBP and p300 (Fig. 2, A and B). Importantly, a

ChIP assay confirmed the association of Ngn1 and MASH1 with the E-box motifs on the PK2 promoter *in vivo* in the OB (Fig. 2, C–E). These data suggested that Ngn1 and MASH1 could regulate the expression of the PK2 gene by binding the E-boxes on its promoter.

We further studied the regulation of Ngn1 and MASH1 on the PK2 gene by examining the expression of the PK2 gene in the OB of *Ngn1*<sup>-/-</sup> and *Mash1*<sup>-/-</sup> mice. *In situ* hybridization with <sup>35</sup>S-labeled probe showed that the expression of the PK2 gene in the OB of P0 *Ngn1*<sup>-/-</sup> mice was reduced to about 30% of the level in WT controls (Fig. 3A,  $p < 0.001$ ,  $n = 4$ ). In the OB of E16.5 *Mash1*<sup>-/-</sup> mice, the expression of the PK2 gene was decreased to about 50% of WT controls (Fig. 3B,  $p < 0.01$ ,  $n = 3$ ). These results indicated that both Ngn1 and MASH1 are critical for the expression of the PK2 gene in the OB and that the residue expression of the PK2 gene in *Ngn1*<sup>-/-</sup> or *Mash1*<sup>-/-</sup> mice might result from redundant activation of PK2 promoter by the other transcription factor.

We have previously shown that PK2 functions as a chemoattractant for the migration of the progenitors for OB interneurons in adult mice (19). As PK2 has also been shown to promote the differentiation of hematopoietic stem cells (17, 27), we examined whether exogenous PK2 could affect the differentiation of neural stem cell. When cultured at low density (200 cells/well in a 96-well plate) in serum-free medium supplemented with 50 nM PK2, neural progenitors from the SVZ of adult mouse did not form neurosphere, indicating that PK2 cannot promote the self-renewal and proliferation of neurosphere-forming cells (Fig. 4, A and D). In addition to that, there was a great reduction in the number of neurospheres formed when PK2 was added to the medium with EGF (20 ng/ml) (Fig. 4D,  $96 \pm 13$  neurospheres/well in medium with EGF versus  $35 \pm 3$  neurospheres/well in medium with EGF and PK2). Moreover, most of the neurospheres formed when PK2 was presented were much smaller than the control group (Fig. 4, B, C, and E). These data suggested that PK2 could antagonize the effect of the mitogen and induce neural progenitors to exit the proliferation state. Interestingly, we saw that many progenitors stopped dividing after the first round division and thus resulted in pairs of sister cells when only PK2 was added to the medium (Fig. 4A).

We then examined the fate of the newly generated cells by *in vitro* differentiation assay. After 7 days of culture, the percentage of  $\beta$ -tubulin III (Tuj1)-positive cells, a marker for immature neurons, nearly doubled from 3.6 to 6.5% and 6.8% when 10 or 50 nM PK2 was added to the medium, comparable with the



**FIGURE 4. PK2 promotes the neuronal differentiation of neural progenitors.** A–C, images of the neurospheres formed after 7 days of culture under different conditions. Scale bar = 20  $\mu$ m. D, quantification of the neurospheres formed under different conditions. \*,  $p < 0.01$ ,  $t$  test,  $n = 24$ . NBA, Neurobasal-A. E, analysis of the size of the neurospheres formed in medium with or without PK2. Most of the neurospheres formed when PK2 was present were much smaller than control group. 50 nM PK2 and 20 ng/ml EGF were used in all neurosphere assays. F, PK2 enhanced the differentiation of neural progenitors into TuJ1-positive cells after 7 days of culture. FBS, fetal bovine serum; RA, retinoid acid. \*,  $p < 0.01$ ,  $n = 3$ . G, PK2 enhanced the differentiation of neural progenitors into MAP2ab-positive cells after 14 days of culture. \*,  $p < 0.01$ ,  $n = 3$ .

neurogenic effect of 1  $\mu$ M retinoid acid (Fig. 4F). After 14 days of culture, the percentage of mature neurons (using MAP2ab as marker) increased substantially when PK2 was added to the medium (Fig. 4G). Taken together, our studies indicated that PK2 could enhance the differentiation of neural progenitors into neurons.

Deficiency of the *PK2* gene leads to an abnormal OB architecture in adult mice, particularly the GCL and PGL (19). Examination of the development of OB in prenatal *PK2*<sup>−/−</sup> mice showed evidently smaller OBs at E16 and E18 when compared with that of WT embryos (supplemental Fig. 3). Cresyl violet staining of the OB of P0 *PK2*<sup>−/−</sup> mice revealed a reduction of the GCL and an abnormal MCL. The reduction of the OB size in *PK2*<sup>−/−</sup> mice was slightly more severe than that of *Ngn1*<sup>−/−</sup> mice (supplemental Fig. 3) and *Mash1*<sup>−/−</sup> mice (28) (data not shown). We further analyzed one population of PGL interneurons in *PK2*<sup>−/−</sup> and *Ngn1*<sup>−/−</sup> mice. Alterations in the development of the tyrosine hydroxylase

(TH)-positive interneurons were observed in the OB of both *PK2*<sup>−/−</sup> and *Ngn1*<sup>−/−</sup> mice at P0. In the OB of *Ngn1*<sup>−/−</sup> mice, many TH-positive cells did not reach their destination in the PGL, but instead, were stalled in the GCL, demonstrating a defect in the migration of these interneurons. However, in the OB of *PK2*<sup>−/−</sup> mice, almost all TH-positive cells were stalled in the GCL, demonstrating a more severe defect in their migration (Fig. 5). The relatively moderate migration defect of TH-positive interneurons in *Ngn1*<sup>−/−</sup> mice might result from the residue expression of PK2 in the OB, which could still attract some of the interneurons into the PGL. The diminished expression of the *PK2* gene in *Ngn1*<sup>−/−</sup> and *Mash1*<sup>−/−</sup> mice and similar defects in OB interneurons in the mutant mice implied that *PK2* is a downstream target gene of *Ngn1* and *MASH1* in the development of the OB.

Proneural bHLH factors are pivotal regulators of the development of nervous system. The known target genes of bHLH factors, most of which are transcription factors, act in cascades to regulate the proliferation, cell type determination, and differentiation of neural progenitors, as well as the migration of neurons to their destination (8, 9). A recent study finds that proneural bHLH factors enhance the radial migration of cortical neurons by regulating the expressions of *RhoA*, *Dcx*, and *p35*, genes critical for the intrinsic cell migration machinery

(14). In this study, we provided evidence that PK2, a ligand for G-protein-coupled receptors, is a functional target gene of *Ngn1* and *MASH1*. Our finding implied that *Ngn1* and *MASH1* can also regulate the migration of olfactory interneurons by controlling the production of PK2, a chemoattractant secreted by OB neurons. This is a novel mechanism that has not been reported previously, suggesting that proneural bHLH factors can regulate cell migration by controlling extracellular signaling in addition to intrinsic migration machinery.

In addition to functioning as a chemoattractant for the migration of OB interneuron progenitors, we found that PK2 can induce neural progenitors to exit proliferation and promote their differentiation into neurons. The expression of PK2 in the olfactory ventricle at E13, when the generation of mitral cells peaks, suggests that PK2 might also be involved in the development of mitral cells. Indeed, we observed an abnormal MCL in the OB of *PK2*<sup>−/−</sup> mice (supplemental Fig. 3). A further study of the defect of mitral cells in



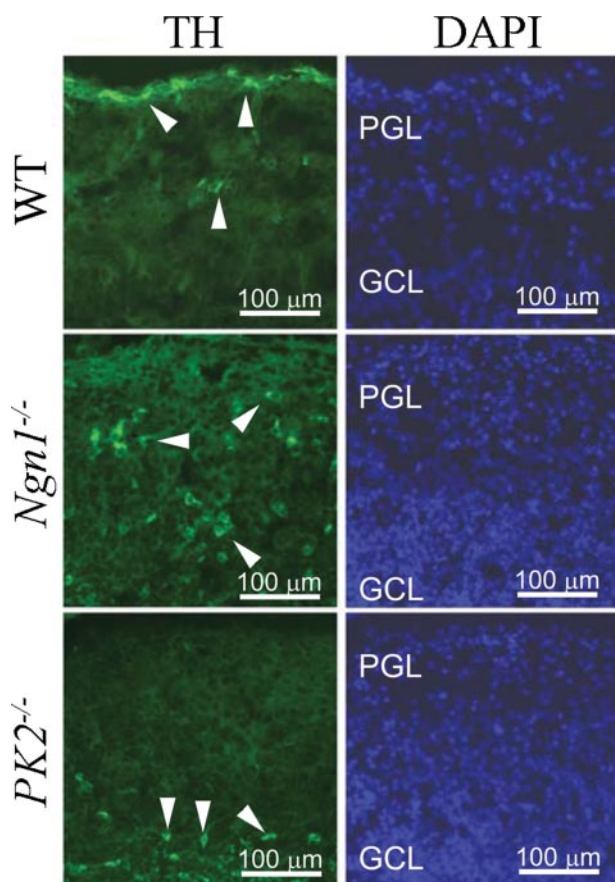


FIGURE 5. Defect of the migration of tyrosine hydroxylase-positive interneurons in *PK2* and *Ngn1* null mice. TH-positive cells were indicated with an arrowhead. DAPI, 4',6-diamidino-2-phenylindole.

the OB of *PK2*<sup>-/-</sup> mice would shed light on the effect of PK2 on mitral cell development. Taken together, our data suggest that PK2, as a functional target gene, appears to mediate some roles of *Ngn1* and *MASH1* in the OB neurogenesis.

Previously, we have demonstrated that *PK2* gene is a functional target of another pair of bHLH factors, *CLOCK* and *BMAL1*, in the regulation of mammalian circadian clock (18). Intriguingly, the same set of E-boxes on the *PK2* promoter appears to be utilized by both groups of bHLH factors. It has also been suggested that PK2 expression might be controlled by hypoxia-inducible factor 1 $\alpha$ , another bHLH factor and key mediator for angiogenesis under hypoxia conditions (16). Our results thus establish *PK2* as a common functional target gene for different bHLH factors in regulating their respective functions (supplemental Fig. 4). Our studies also provide an example that the complexity of mammalian genome is probably not due to a sheer increase in the quantity of genes but most likely to complicated gene regulation networks (29).

**Acknowledgment**—We thank Dr. Michael Greenberg for providing anti-*Ngn1* antibodies.

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